EXHIBIT 2

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by The University of Queensland

EXHIBIT SMT-2

This is Exhibit SMT-2 referred to in the Statutory Declaration by Stephen Maxwell Taylor

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Before me:

A person empowered to witness Statutory Declarations under the laws of the Queensland, Commonwealth of Australia

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Antiarthritic Activity of an Orally Active C5a Receptor Antagonist Against Antigen-Induced Monarticular Arthritis in the Rat

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Objective. To determine if the new, orally active C5a receptor antagonist, the cyclic peptide AcF-[OPdChaWR], reduces the severity of pathology in a rat model of immune-mediated monarticular arthritis.

Methods. Arthritis was induced in the right knee of previously sensitized rats by the intraarticular injection of methylated bovine serum albumin. Rats were examined for either 14 days or 28 days, or for 49 days following a second antigen challenge at 28 days. The C5a antagonist (1 or 3 mg/kg/day) and/or ibuprofen (30 mg/kg/day) were administered orally on a daily basis either before or after arthritis induction.

Results. Rats receiving AcF-[OPdChaWR] had significant reductions in right knee swelling, gait disturbance, lavaged joint cell numbers, and right knee histopathology, as well as in serum levels of tumor necrosis factor α (TNFα) and intraarticular levels of interleukin-6 and TNFα on day 14. In the 14- and 28-day studies, ibuprofen resulted in a similar reduction in gait abnormalities and intraarticular inflammatory cells compared with the C5a antagonist, but was less effective in reducing knee swelling over the course of the study and had no effect on knee histopathology. Combination therapy with AcF-[OPdChaWR] and ibuprofen resulted in no greater efficacy than with the C5a antagonist alone. Rats injected twice with the antigen in the 49-day study displayed the most severe histopathology and this, as well as knee swelling and gait abnormalities, was significantly reduced by repeated treatment with the C5a antagonist.

Conclusion. An agent that inhibits the action of C5a in this model significantly reduced joint pathology, while ibuprofen was not effective. C5a antagonists could therefore have broader therapeutic benefits than nonsteroidal antiinflammatory drugs as antiarthritic agents for rheumatoid arthritis.

Complement activation produces the 74-residue protein C5a, which is the most potent of the anaphylatoxins (1). C5a mediates numerous immune and inflammatory functions, including chemotaxis and activation of inflammatory cells, increased vascular permeability, spasmogenesis, immune regulation, and the release of a variety of inflammatory cytokines and mediators (1–3).

Rheumatoid arthritis (RA) is an immune complex disease involving the local activation of inflammatory cells, predominantly in the smaller peripheral joints. The complement system, and in particular the factor C5a, has long been identified as a likely contributor to the pathogenesis of RA. Elevated levels of C5a have been found in the plasma and inflamed joints of patients with RA (4,5). The number of C5a receptors on synovial mast cells is also increased in RA (6). Most important, the degree of complement activation in the joint and circulation correlates with the severity of the disease, suggesting that C5a plays a central role in disease pathogenesis (7–10).

Several animal models of RA have been used to elucidate the role of complement and C5a in this

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disease. Depletion of complement with cobra venom factor has been shown to delay the disease onset in a number of laboratory models of RA, including adjuvant arthritis, collagen-induced arthritis (CIA), and antigen-induced arthritis (AIA) in rats (11–13). Administration of recombinant soluble complement receptor 1 (sCR1) or anti-C5 antibodies, either intraperitoneally (IP), intravenously (IV), or intraarticularly (IA), has been shown to reduce disease progression in anti-CD59 antibody–induced models of acute arthritis (14), CIA (13,15), and AIA (12). However, the role of C5a as a pathogenic factor in these models has been difficult to prove due to the lack of availability of a specific C5a antagonist.

This issue was addressed recently with the use of a peptidic C5a receptor antagonist, MeFKPdChaWr, in a model of membrane attack complex (MAC)–dependent anti-CD59 antibody-induced arthritis (14). Administration of the antagonist, either IV or IA, failed to inhibit the development of disease parameters (14). Explanations for the apparent lack of C5a involvement in this animal model include the possibility of impaired clearance of MAC rather than activation of the complement cascade, failure of pathology to be affected by complement depletion, and resolution of the lesion within 3 days (16).

Current drug therapies for RA remain relatively ineffective at retarding disease progression and are associated with significant side effects (for review, see ref. 17). Drugs such as the nonsteroidal antiinflammatory drugs (NSAIDs), which include ibuprofen, provide palliative relief of symptoms, but have little beneficial effect on the pathology and progression of disease (17,18). Patients with developing RA require an effective, orally active therapy that will moderate the symptoms as well as arrest the progression of the destructive joint pathology.

Researchers in our laboratory have recently developed a series of small molecule antagonists of the human C5a receptor (19,20). One of these is a cyclic peptide, AcF-[OPdChaWR], which is orally active and has been shown to effectively reduce the severity of diseases in various rat models (21–23). In the present study, we tried to use this specific C5a receptor antagonist to determine the relative contribution of C5a in the pathogenesis of immune-mediated monarticular arthritis and assess the effectiveness of the antagonist in treating the disease symptoms and pathology. This compound has been shown to be a potent antagonist of both human and rat C5a receptors on polymorphonuclear leukocytes (PMNs) (24). It is also a potent antagonist of C5a receptors on human macrophages (25). We therefore hypothesized that any demonstration of antiarthritic activity in rats would bode well for future testing in human arthritic conditions. The data herein demonstrate that oral administration of AcF-[OPdChaWR], either before or after induction of the disease, is effective at preventing the development of pathology and moderating disease progression. These findings confirm the importance of C5a in this disease model and suggest a future role for antagonists of the C5a receptor in the treatment of immune-mediated arthritis and other inflammatory diseases.

MATERIALS AND METHODS

Animals. Female Wistar rats weighing 225–275 gm were used in this study. All animal experimentation conducted in this study was performed in accordance with National Health and Medical Research Council of Australia guidelines.

Antagonist preparation. The cyclic peptide, AcF-[OPdChaWR] (AcPhe[Orn-Pro-D-cyclohexylalanine-Trp-Arg]), was manufactured as previously described (20). The compound was purified by reverse-phase high-performance liquid chromatography and fully characterized by mass spectrometry and proton nuclear magnetic resonance spectroscopy.

Experimental procedure. Rats were sensitized by subcutaneous injection of 0.5 mg methylated bovine serum albumin (mBSA; Sigma, St. Louis, MO) in 0.5 ml Freund's complete adjuvant (Sigma) on day 21 and day 14 prior to challenge. Two weeks after the second injection (day 0), rats were anesthetized with ketamine (80 mg/kg IP, ilium; Lyppard, Brisbane, Australia) and xylazine (12 mg/kg IP, ilium; Lyppard) and both hind legs were shaved. A suspension of 0.5 mg mBSA in 100 μl saline was aseptically injected into the joint space of the right knee, with the contralateral left knee receiving saline alone. Swelling of the right and left knees was quantitatively assessed at various times during the study period by measuring the medial-lateral width across the joint with Vernier calipers. The appearance of each rat's gait was also scored (0–4) independently in a blinded manner during the study. A normal gait (no limp and full weight-bearing ability on the right hind leg) was scored as 0; a mild limp on the right hind leg was scored 1; a moderate limp on the right hind leg was scored 2; a severe limp on the right hind leg was scored 3; and no weight-bearing ability on the right hind leg was scored 4.

At the completion of each study, left and right knee joints were lavaged with 100 μl saline and total and differential cell counts were performed. The fluid was then centrifuged (11,000g, 3 minutes) and the supernatant stored at −20°C until analyzed for concentrations of tumor necrosis factor α (TNFα) and interleukin-6 (IL-6). A sample of blood was also obtained, allowed to clot, and centrifuged (11,000g, 3 minutes), with the resulting serum stored at −20°C for later determination of TNFα concentrations. The knee joints from every rat were dissected out, stored in 10% buffered formalin for at least 10 days, and decalcified in a saturated solution of EDTA for 21

days. They were then embedded in paraffin, and sections were cut, mounted, and stained using hematoxylin and eosin.

Study design and treatment groups. Three separate experimental trials were conducted lasting either 14, 28, or 49 days after arthritis induction. The trials lasting 14 or 28 days were performed twice and data were pooled for the final analysis. There were no differences in results between the replicate experiments. A very small proportion of sensitized rats (4 of 130) showed no knee swelling after intraarticular injection of antigen; these rats were excluded from the study.

In the initial experimental trial, rats were monitored for 14 days after arthritis induction. One group of rats received 1 mg/kg/day AcF-[OPdChaWR] in the drinking water, commencing 2 days prior to the induction of arthritis on day 0 and continuing throughout the study. The control group received water only. It was initially determined that rats drink 25 ± 3 ml water per day (mean ± SEM; n = 8), and drinking volumes were monitored daily during the experiments to validate the dose delivery. Administration of the C5a antagonist at this dose in the drinking water of rats for 7 days was effective at completely blocking C5a-induced neutropenia and the binding of C5a to neutrophils isolated from these rats (data not shown). The prolonged in vivo activity of the C5a antagonist following a single oral dose (22) also validated this dose regimen. Another group of rats received either AcF-[OPdChaWR] (1 mg/kg/day) or ibuprofen (sodium salt, 30 mg/kg/day; Sigma) in the drinking water from day +2 onward, with control rats receiving water alone.

In the second study, rats were monitored for 28 days after arthritis induction. Animals received either AcF-[OPdChaWR] (1 mg/kg/day), ibuprofen (sodium salt, 30 mg/kg/day), or a combination of AcF-[OPdChaWR] (1 mg/kg/day) and ibuprofen (sodium salt, 30 mg/kg/day) in the drinking water from day −2 onward. Control animals received water only. A separate group of rats received AcF-[OPdChaWR] (3 mg/kg/day) in the drinking water from day +4 onward, with control rats receiving water only.

In the third study, rats were monitored for 49 days after induction of arthritis, with rats being reanesthetized on day 28 with a second injection of 0.5 mg mBSA in 100 μl saline in the right knee and saline alone in the left knee. In this study, one group of rats received 1 mg/kg/day AcF-[OPdChaWR] in the drinking water from day −2 and throughout the study, with the arthritic control rats receiving water only.

TNFα and IL-6 measurement. Samples of serum and intraarticular lavage fluid were assayed for TNFα levels using an enzyme-linked immunosorbent assay (ELISA) kit (PharMingen, San Diego, CA) with a 1:10 dilution of samples as previously described (21,22). Intraarticular levels of IL-6 in the lavage fluid were determined using an ELISA method as previously described (21,22). Concentrations of TNFα and IL-6 in the samples were determined by linear regression analysis from the standard curve.

Histologic evaluation. Histologic sections from the right and left stifle joints of each animal were examined by an independent observer (IAS) in a blinded manner and graded on a scale of 0–4. A grade of 0 involved no detectable abnormalities. Pathology of grade 1 had some inflammatory cell infiltration in the synovial membrane with no significant thickening of the membrane or cartilage erosion. Joints with extensive inflammatory cell infiltration and thickening of the synovial membrane were scored 2. Joints with a more severe lesion were scored as 3, while the most severe lesion (scored 4) showed significant thickening and fibrosis of the joint capsule, involvement of the articular cartilage, appearance of inflammatory cells in the joint space, and extensive synovitis.

Statistical analysis. All experimental results are expressed as the mean ± SEM. Data were analyzed using GraphPad Prism 3.02 software (GraphPad Software, San Diego, CA). Statistical comparisons were made between drug-free arthritic rats and all other treatment groups or between the arthritic right knees and saline-injected left knees, using either Student's *t*-test or the Mann-Whitney U test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Fourteen-day study. Measurements of the saline-injected left knee of each rat did not significantly change from preinjection values during the course of each experiment (data not shown). Following the injection of mBSA on day 0 in drug-free sensitized rats, the average increase in the width of the right knee peaked on day +3 (4.69 ± 0.32 mm; n = 11) (Figure 1A). Rats that had been pretreated from day −2 with AcF-[OPdChaWR] (1 mg/kg/day) had significantly ($P < 0.05$) reduced right knee widths from days +2–14 (peak on day +3 2.08 ± 0.59 mm; n = 9) compared with drug-free arthritic rats (Figure 1A). Gait scores in drug-free arthritic rats also increased above baseline levels following induction of arthritis (Figure 1B). Pretreatment with the C5a antagonist significantly decreased these scores from days +2–14 ($P < 0.05$). There was a high correlation between gait scores and knee swelling in drug-free arthritic rats for all 3 experimental trials (14-day trial $r^2 = 0.83$, n = 7; 28-day trial $r^2 = 0.96$, n = 11; 49-day trial $r^2 = 0.84$, n = 17) from day +2 to completion of the study.

In a separate study, the effects of the C5a antagonist or ibuprofen on knee swelling and gait scores following the establishment of arthritis were examined. Rats treated with either AcF-[OPdChaWR] (1 mg/kg/day) or ibuprofen (30 mg/kg/day) from days +2–14 had significantly reduced knee swelling (days +3–14) and gait scores (days +4–14) compared with arthritic rats that received no drug treatment ($P < 0.05$; Figures 1C and D).

In the initial 14-day study, TNFα and IL-6 levels were found to be elevated in the right knee lavage fluid on day 14, as were TNFα levels in the serum of drug-free arthritic rats (Figures 2A and B). Rats pretreated with the C5a antagonist had significantly lower levels of these cytokines ($P < 0.05$; Figures 2A and B) in the joint and serum on day 14. The majority (>90%) of cells recovered from the right knee lavage fluid on day 14 were

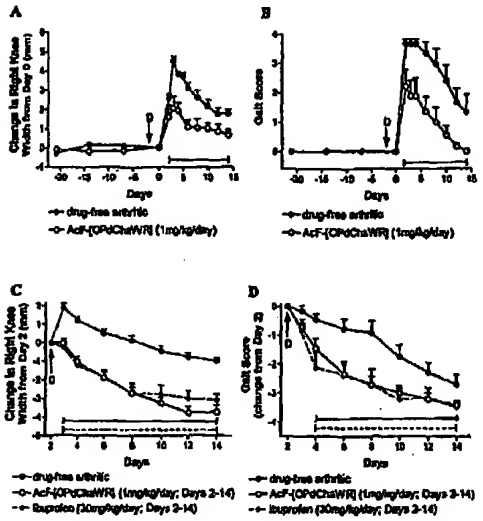

Figure 1. Right knee swelling and gait scores (14-day study). Antigen was injected into the right articular capsule of rats on day 0, which resulted in increases in knee swelling and gait scores (scored 0–4). Rats treated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 onward had significantly reduced knee swelling (A) and gait scores (B) compared with drug-free arthritic rats. Rats treated with AcF-[OPdChaWR] (1 mg/kg/day) or ibuprofen (30 mg/kg/day) from day +2 onward also had a significant reduction in knee swelling (C) and gait scores (D) compared with drug-free arthritic rats. Results are expressed as the mean and SEM, with periods of significant difference from drug-free arthritic rats denoted by bars (solid for AcF-[OPdChaWR] and dashed for ibuprofen) ($P < 0.05$; n = 9–12). D = period when drug treatment began.

PMNs. Significantly fewer PMNs were found in the lavage fluid of rats treated with AcF-[OPdChaWR] or ibuprofen throughout the study, or in rats treated with the C5a antagonist from day +2, compared with drug-free arthritic rats ($P < 0.05$; Figure 2C).

The saline-injected left knees of all rats in every study showed no histologic abnormalities (Figure 3A) and all were scored 0 (data not shown). Sections from the right knees of drug-free arthritic rats on day 14 had marked cellular infiltration, which was predominantly neutrophils, and mild synovial cell proliferation, with a mean ± SEM histopathology score of 3.2 ± 0.3 (n = 16; Figures 2D and 3B and C). Histologic sections from rats pretreated with AcF-[OPdChaWR] had a lesser degree of cellular infiltration and synovial proliferation, resulting in a significantly lower histopathologic score of 1.4 ± 0.5 ($P < 0.05$; n = 8) (Figures 2D and 3D and E). Histologic sections from rats posttreated with the C5a antagonist 2 days after the induction of arthritis also had a lower degree of cellular infiltration and synovial proliferation compared with those from drug-free arthritic rats, although to a lesser extent than rats pretreated on day −2 with the C5a antagonist (Figure 3F).

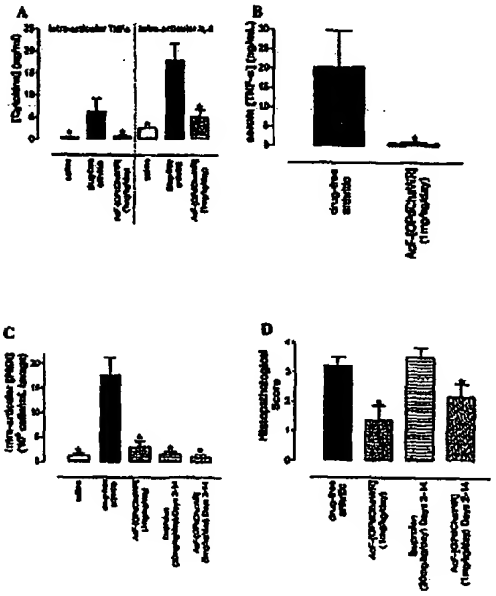

Figure 2. Intraarticular cytokine and polymorphonuclear leukocyte (PMN) levels, serum tumor necrosis factor α (TNFα) levels, and histopathologic scores (14-day study). Antigen was injected into the right articular capsule and saline into the left articular capsule of rats on day 0. On day 14, the animals were killed and the knee capsules lavaged for determination of TNFα and interleukin-6 (IL-6) levels, and PMN numbers. Serum was also analyzed for TNFα concentrations, and histopathologic analysis (scored 0–4) was performed on knee sections. A, Intraarticular TNFα and IL-6 concentrations were found to be significantly reduced in the right knee capsules of rats treated with AcF-[OPdChaWR] (1 mg/kg/day, days −2–14) compared with drug-free arthritic rats (n = 4–8). B, Serum levels of TNFα were also significantly reduced in rats treated with AcF-[OPdChaWR] (1 mg/kg/day, days −2–14) compared with drug-free arthritic rats (n = 8). C, Numbers of PMNs in the right knee intraarticular lavage fluid were significantly reduced in all drug treatment groups compared with right knee articular fluid from the drug-free arthritic rats (n = 8–12). D, Histopathologic scores in rats treated with AcF-[OPdChaWR] (1 mg/kg/day), either from days −2–14 or from days +2–14, were significantly lower than those in drug-free arthritic rats (n = 8–16). Rats treated with ibuprofen (30 mg/kg/day, days +2–14) had no improvement in histopathologic scores (n = 5). Data are expressed as the mean and SEM. * = $P < 0.05$.

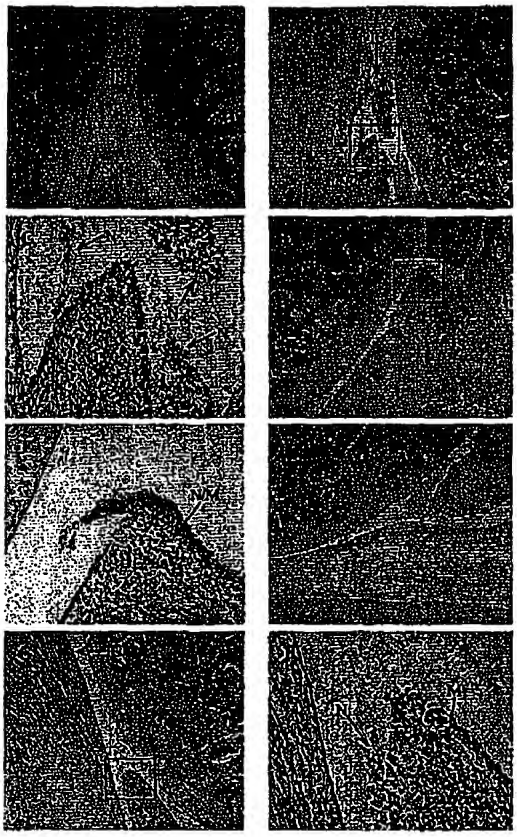

Figure 3. Sections of knees from rats in the 14-day study. A, Saline-injected left knee with normal synovial membrane (SM) and joint space (JS) (score 0). B, Arthritic right knee of a drug-free rat, with inflammatory cells (IC) evident in the joint space (score 3). C, Higher magnification of the boxed area in B, with neutrophils (N) indicated. D, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day, days −2–14)–treated rat (score 1). E, Higher magnification of the boxed area in D, with neutrophils and macrophages (M) shown. F, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day, days +2–14)–treated rat (score 1). G, Right knee of an ibuprofen (30 mg/kg/day, days +2–14)–treated rat (score 2.5). H, Higher magnification of the boxed area in G, with neutrophils and macrophages indicated. S = areas of synovitis. Images are typical and representative of each treatment group, and the score indicated is for the section shown. (Hematoxylin and eosin stained; original magnification ×40 in A, B, D, F, and G; ×200 in C, E, and H.)

The mean histopathologic score in these posttreated rats was 2.2 ± 0.4 (n = 8), which was significantly lower than that in drug-free arthritic rats ($P < 0.05$; Figure 2D). In contrast, histologic sections from rats treated with ibuprofen (30 mg/kg/day) from days +2–14 had no change in parameters (score 3.5 ± 0.3; n = 6) compared with those from drug-free arthritic rats (Figures 2D and 3G and H).

Twenty-eight–day study. Following arthritis induction, right knee widths of drug-free arthritic rats rapidly increased above baseline to peak on day +3 (4.98 ± 0.39 mm above baseline; n = 14) and slowly decreased to 0.51 ± 0.21 mm on day 28 (Figure 4A). Rats pretreated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 had significantly lower right knee widths

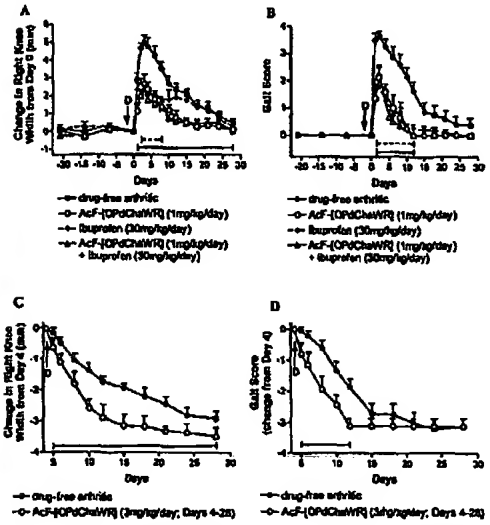

Figure 4. Right knee swelling and gait scores (28-day study). Antigen was injected into the right articular capsule of rats on day 0, which resulted in increases in knee swelling and gait scores (scored 0–4). Rats treated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 onward had significantly reduced knee swelling (A) and gait scores (B) compared with drug-free arthritic rats. Rats treated with ibuprofen (30 mg/kg/day, days −2–28) also had significantly reduced knee swelling (A) and gait scores (B) compared with drug-free arthritic rats, but for a lesser time period than rats treated with the C5a antagonist for knee swelling. Rats treated with a combination of both the C5a antagonist (1 mg/kg/day) and ibuprofen (30 mg/kg/day) from day −2 onward also had significant reduction in these parameters (A and B), which was comparable with those in rats treated only with the C5a antagonist. Rats treated with AcF-[OPdChaWR] (3 mg/kg/day) from day +4 onward again had a significant reduction in knee swelling (C) and gait scores (D) compared with drug-free arthritic rats. Results are expressed as the mean and SEM, with periods of significant difference from drug-free arthritic rats denoted by bars (dashed for ibuprofen and solid for AcF-[OPdChaWR] and AcF-[OPdChaWR]/ibuprofen combination) ($P < 0.05$; n = 11–16). D = period when drug treatment began.

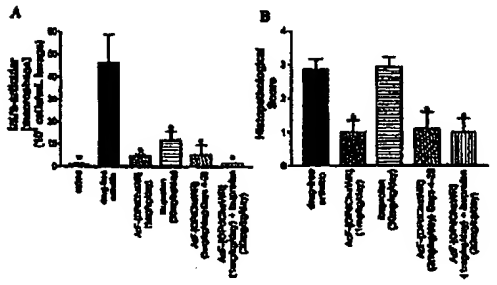

Figure 5. Intraarticular macrophages and histopathologic scores (28-day study). Antigen was injected into the right articular capsule and saline into the left articular capsule of rats on day 0. On day 28, the animals were killed, knee capsules were lavaged for determination of macrophage numbers, and histopathologic analysis (scored 0–4) was performed on stained knee sections. A, Macrophage numbers in the right knee intraarticular lavage fluid were significantly reduced in all drug treatment groups compared with right knee articular fluid from the drug-free arthritic rats (n = 6–10). B, Histopathologic scores from rats treated with AcF-[OPdChaWR] either from days −2–28 (1 mg/kg/day) or from days +4–28 (3 mg/kg/day), or in rats treated from days −2–28 with a combination of the C5a antagonist (1 mg/kg/day) and ibuprofen (30 mg/kg/day) were significantly lower than those from drug-free arthritic rats (n = 10–16). Rats treated with ibuprofen alone (30 mg/kg/day, days −2–28) had no improvement in histopathologic scores (n = 6). Data are expressed as the mean and SEM. * = $P <$ 0.05.

from days +1–28 (peak on day +3 2.66 ± 0.52 mm; n = 12) ($P < 0.05$; Figure 4A). Rats pretreated with ibuprofen (30 mg/kg/day) had significantly decreased knee widths from days +2–8 only (peak on day +3 2.30 ± 0.30 mm; n = 11) ($P < 0.05$; Figure 4A). Rats receiving a combination of the C5a antagonist (1 mg/kg/day) and ibuprofen (30 mg/kg/day) from day −2 had a significant reduction in right knee widths from days +1–28 (peak on day +3 2.19 ± 0.06 mm, n = 12) ($P < 0.05$; Figure 4A), which was comparable with that of rats receiving the C5a antagonist alone. Gait scores in rats pretreated with either the C5a antagonist, ibuprofen, or the combination of both were significantly reduced compared with those of drug-free arthritic rats from days +1–12 ($P < 0.05$; Figure 4B).

In the 14-day studies, which were performed first in the series reported here, the C5a antagonist was administered at 1 mg/kg/day both as a preventative (days −2–14) and as reversal (days +2–14) therapy. It was noted that rats treated with the antagonist at this dose from day +2 had less improvement in histopathologic scores compared with rats treated from day −2 (Figure 2D). In the following 28-day reversal studies, a higher dose of C5a antagonist (3 mg/kg/day) was used to determine if this might be a more effective reversal dose regimen. It was found that rats treated with this dose regimen had significantly improved right knee widths (days +5–28) and gait scores (days +5–12) compared with drug-free arthritic rats ($P < 0.05$; Figures 4C and D).

In all rats in the 28-day studies, there were no detectable levels of TNFα, either in the serum or in the knee lavage fluid, on day 28. The vast majority (>95%) of cells recovered in the lavage fluid of the right knees of rats were macrophages. Drug-free arthritic rats had an average of 46.5 ± 12.3 × 10^4 macrophages/ml lavage fluid (n = 10; Figure 5A), an ~20-fold increase over saline-injected left knees. Rats pretreated with AcF-[OPdChaWR], ibuprofen, or a combination of both, or rats treated from day +4 with the C5a antagonist alone, had significantly lower numbers of macrophages in the right knee lavage fluid (Figure 5A).

Histologic analysis of the right knees of arthritic drug-free rats revealed varying degrees of synovial cell proliferation and cellular infiltration, and these were scored overall, with an average of 2.9 ± 0.3 (n = 16; Figures 5B and 6A and B). Sections from rats either pretreated with C5a antagonist (1 mg/kg/day) or treated from day +4 (3 mg/kg/day) had an equal reduction in severity of lesions compared with drug-free arthritic rats, with significantly decreased scores of 1.0 ± 0.4 and 1.1 ± 0.5, respectively (n = 12 in each group) ($P < 0.05$; Figures 5B and 6C and H). Conversely, rats pretreated with ibuprofen (30 mg/kg/day) had no improvement in right knee pathology, with an average score of 2.9 ± 0.3 (n = 6; Figures 5B and 6D and E). Rats pretreated with a combination of the C5a antagonist and ibuprofen also had a significant reduction in histopathologic scores, which was similar to that in rats treated with only the C5a antagonist (n = 12; Figures 5B and 6F and G).

Forty-nine–day study. In drug-free rats following the first injection of mBSA on day 0, the right knee increased in width, with a peak swelling above baseline of 5.27 ± 0.56 mm (n = 6) on day +3. Following the second injection of mBSA on day 28, the increase in width of the right knee was similar in magnitude to the first challenge, with a peak of 4.47 ± 0.60 mm above preinjection values 1 day after the second injection (Figure 7A). Rats pretreated with AcF-[OPdChaWR] at 1 mg/kg/day had significantly lower right knee widths compared with drug-free rats from days +1–49, with peaks of 2.87 ± 0.51 mm above baseline (day 3) and 2.64 ± 0.37 mm (day 29) for the first and second injections, respectively (n = 6) ($P < 0.05$; Figure 7A).

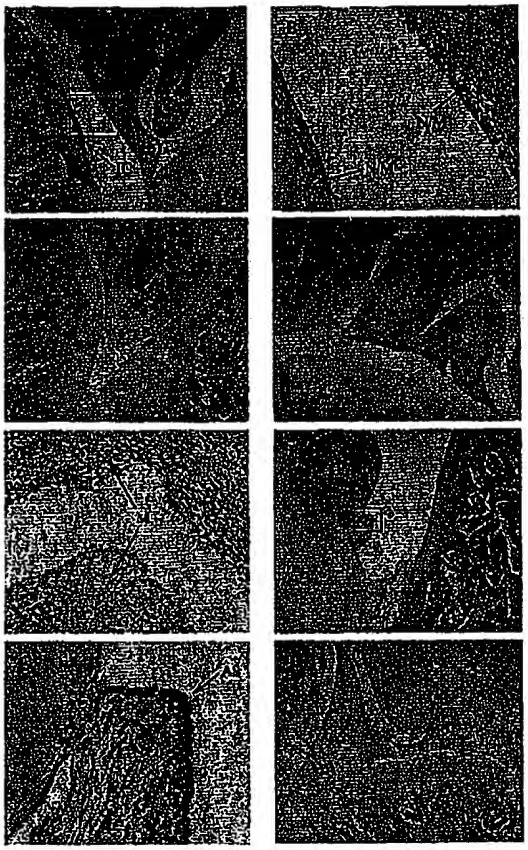

Figure 6. Sections of knees from rats in the 28-day study. A, Arthritic right knee of a drug-free rat, with inflammatory cells (IC) lining the cartilage and areas of synovial fibrosis (F) indicated (score 2.5). B, Higher magnification of the boxed area in A, with neutrophils (N) and macrophages (M) evident. C, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day, days −2–28)–treated rat (score 0); D, Right knee of an ibuprofen (30 mg/kg/day, days −2–28)–treated rat (score 3). E, Higher magnification of the boxed area in D, with macrophages shown. F, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day) + ibuprofen (30 mg/kg/day) combination (days −2–28)–treated rat, with the patella (P) indicated (score 1). G, Higher magnification of the boxed area in F, with activated synoviocytes (AS) shown. H, Right knee of an AcF-[OPdChaWR] (3 mg/kg/day, days +4–28)–treated rat (score 0). S = areas of synovitis. Images are typical and representative of each treatment group, and the score indicated is for the section shown. (Hematoxylin and eosin stained; original magnification ×40 in A, C, D, F, and H; ×200 in B, and E; × 400 in G.)

Gait scores increased in drug-free arthritic rats, with the deterioration in gait proportional to right knee widths, and maximum gait scores following both the first and the second injection (Figure 7B). C5a antagonist–dosed rats had significantly improved gait scores from days +2–16 and +30–44 ($P < 0.05$; Figure 7B).

Examination of right knee sections from 49-day arthritic drug-free rats showed a more severe pathology than was seen in the other studies involving single intraarticular injection of antigen and more limited experimental time spans. All antigen-injected right knees in drug-free rats had marked inflammatory cell infiltration and severe synovial proliferation and fibrosis (Figures 8A and B). Additionally, cartilage erosion was observed in all sections from challenged right knees, resulting in an average histopathologic score of 3.9 ± 0.1 (n = 6, Figures 7C and 8A and B). Sections of right knees from rats that had been pretreated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 onward had decreased pathology compared with that of drug-free arthritic rats, with a significantly improved score of 1.7 ± 0.7 (n = 6, Figures 7C and 8C and D).

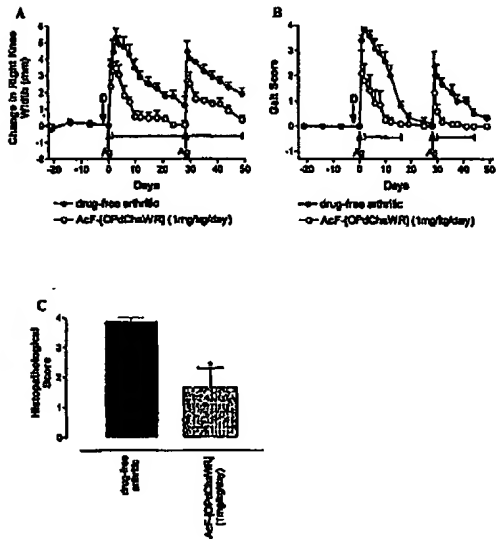

Figure 7. Right knee swelling, gait scores, and histopathologic scores (49-day study). Antigen was injected into the right articular capsule of rats on days 0 and 28, which resulted in increases in knee swelling and gait scores (scored 0–4). Rats treated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 onward had significantly reduced knee swelling (A) and gait scores (B) compared with drug-free arthritic rats. On day 49, animals were killed and histopathologic analysis (scored 0–4) was performed on stained knee sections. Rats treated with AcF-[OPdChaWR] (1 mg/kg/day, days −2–49) had significantly lower scores compared with drug-free arthritic rats (C). Results are expressed as the mean and SEM, with periods of significant difference from drug-free arthritic rats denoted by bars. D = period when drug treatment began; Ag = days antigen was injected. * = $P < 0.05$; n = 6.

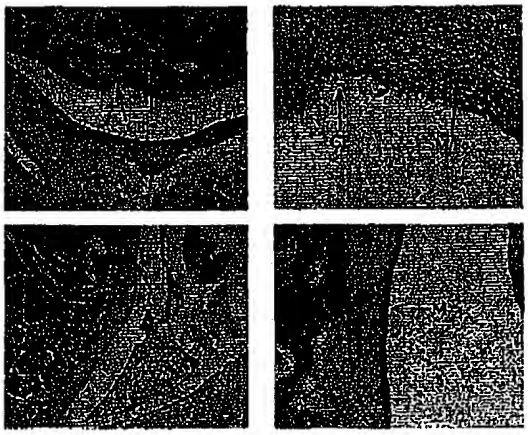

Figure 8. Sections of knees from rats in the 49-day study. A, Arthritic right knee of a drug-free rat, with inflammatory cells (IC) degrading the cartilage and a section of cartilage erosion (CE) shown (score 4). B, Higher magnification of the boxed area in A, with macrophages (M) and fibrocytes (Fc) shown alongside cartilage erosion. C, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day, days −2–49)–treated rat, with mild appearance of inflammatory cells lining cartilage (score 1). D, Higher magnification of the boxed area in C, with macrophages shown. S = areas of synovitis. Images are typical and representative of each treatment group, and the score indicated is for the section shown. (Hematoxylin and eosin stained; original magnification ×40 in A and C; ×200 in B and D.)

DISCUSSION

Current drug treatment of RA involves the inhibition of cytokines and other mediators thought to be involved in its pathogenesis. The complement factor C5a is also recognized as a very important proinflammatory mediator in RA (6). Levels of C5a in the plasma and synovial fluid of patients with RA are higher than those found in patients with osteoarthritis (5). The pathology of RA includes the recruitment and accumulation of neutrophils and monocytes in the synovial tissues, with the down-regulation of C5a receptors in monocytes possibly contributing to chronicity of the disease (26,27). Synovial effusions contain high levels of C5a, which is a powerful chemoattractant for neutrophils and monocytes and invokes microvascular plasma leakage within the tissues (4,26). Inhibitors of activation of the complement system, or of the formation or action of C5a, have therefore been proposed to be of potential use in the treatment of RA. Anti-C5 monoclonal antibodies have been shown to block the development of CIA in rats as well as reduce the progression of established pathology (15). A recombinant protein inhibitor of complement activation, sCR1, has been suggested as a potential therapy for RA because efficacy with this compound has been shown in various animal models of arthritis (12,13,28).

The model of an antigen-induced monarticular Arthus reaction produces a discrete lesion of highly reproducible severity in a single joint, leaving the contralateral joint available for comparison. It has been suggested that the changes in the joint in clinical RA are due to this reaction (29). Neutrophils predominate in the synovial fluid in RA, particularly in the early stages, and this is mirrored in this rat model (30). Mononuclear cells were the predominant inflammatory cells in the rat synovium; this is also the case in RA (31). Additionally, the presence of the proinflammatory cytokines TNFα and IL-6 in the arthritic knee joint and TNFα in the serum of rats in the 14-day study correlates with the presence of these cytokines in the joints of arthritis patients (32).

The findings reported here show that an orally active, small molecule antagonist of the C5a receptor reduces the disease severity in a rat model of immune-mediated arthritis. Given on a daily basis, either before the initiation of arthritis or after symptoms were detectable, the drug reduced both joint swelling and gait symptoms. In the latter treatment case, drug therapy was started either 2 or 4 days after the lesion was stimulated in order to mimic the human clinical situation where a patient would present with an acute progressive lesion. In the 14-day study, PMNs were the predominant cell recovered in joint lavage fluid, whereas in the 28-day study, macrophages were the principal cell type found. In these shorter-term studies, cartilage erosion did not occur. In order to stimulate erosion, it was necessary to prolong the duration of the study and rechallenge the rats with antigen. Under these conditions, the C5a antagonist was again effective in reducing the degree of structural change in the joint.

These results show that this class of drug has multiple activities at different stages of the disease process. In previous short-term studies involving endotoxic shock and the Arthus reaction in the peritoneum and the dermis, the C5a antagonist was very effective at inhibiting the inflammatory challenges (21,22,33). The results of the present study demonstrate the efficacy of this class of drugs in a model of chronic immune complex–mediated inflammation, following daily oral administration of the drug.

The destruction of cartilage in osteoarthritis results from the IL-1–stimulated degradation of proteoglycans and inhibition of chondrocyte proteoglycan synthesis (18). NSAIDs protect the joint from swelling and cellular infiltration, but have little effect on disease

progression, while glucocorticoids normalize proteoglycan synthesis (17,18). The NSAID ibuprofen diminishes the responses in the rat as measured by joint swelling and disturbance of gait, and these findings equate well with the response to most NSAIDs in the clinic (17,34). Ibuprofen is less successful in reducing the structural pathology in the rat joint, and this is also similar to human clinical findings (17,34). In contrast, the C5a receptor antagonist used in this study significantly reduces the degree of structural pathology in the joint as well as other signs of the disease in the rats. This ability to moderate structural changes in the joint is a clear advantage over most of the NSAIDs.

The C5a antagonist used in the present study is orally active, with peak circulating plasma levels around 0.1–0.3 μM following a single oral dose of 3 mg/kg (22). The drug has a high affinity for human C5a receptors, and is an insurmountable antagonist active at low nanomolar concentrations (25). The drug was developed by structural analysis of the effector portion of C5a, and is a cyclic peptide that is resistant to metabolic degradation in the gut and plasma (20). In rats, a single oral dose of 10 mg/kg inhibits the neutropenia response to IV C5a for up to 24 hours (22), and oral treatment for 7 days at a dose of 1 mg/kg completely blocks the binding of C5a to circulating PMNs. In the present study, administration of 1 mg/kg/day was also an effective oral dose for reducing the expression of the arthritis symptoms and joint pathology. This C5a antagonist does not inhibit the formation of MAC at concentrations up to 100 μM (Strachan AJ: unpublished observations), indicating that, at least in the rat model of monarticular arthritis, inhibition of C5a alone is sufficient for effective reduction of symptoms and pathology. Similar results have been found in the same rat model of arthritis for the protein sCR1, which inhibits the formation of C3a, C5a, and MAC (12). This is consistent with our hypothesis that C5a is a major pathogenic mediator of disease pathology in this model. The precise role of MAC in antigen-induced monarticular arthritis, however, remains to be fully established.

In this study, combination drug therapy with ibuprofen and the C5a antagonist was found to be no more effective than therapy with the C5a antagonist alone. C5a causes the release of eicosanoids when administered in vivo to a variety of animal species, and the effects of C5a on blood pressure are blocked by cyclooxygenase inhibitors (35–37). The lack of additional efficacy on all parameters with the combination therapy may be due to the fact that the eicosanoid cascade is blocked early on by the C5a antagonist. The increased efficacy of the C5a antagonist over ibuprofen for joint pathology may be due to the additional inhibition of expression of proinflammatory cytokines, such as TNFα and IL-6. The importance of TNFα in the pathology of RA has been clearly demonstrated in a number of studies and has led to the development of a soluble TNFα receptor–based treatment (etanercept) in RA (38,39). It has previously been shown that inhibitors of C5a reduce the expression of TNFα and IL-6 in other disease models in vivo (21–23,40,41). The ability of the C5a antagonist to inhibit the expression of TNFα indicates an early role for C5a in the inflammatory cascade, and suggests a pivotal role for the therapeutic use of C5a antagonists in RA. The disease-modifying properties of the C5a antagonist in the present study may be due to its capacity to inhibit formation of both these cytokines and eicosanoids, rather than to the inhibition of eicosanoids alone.

In summary, this study demonstrates for the first time that a small molecule C5a receptor antagonist, given orally, prevents some principal signs of arthritis and significantly reduces the joint damage caused by an immune-mediated monarticular arthritis in rats. The disease-modifying effects of this antagonist suggest a potential use for C5a antagonists as antiarthritic agents in the clinic.

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